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Orthopoxvirus Detection in Environmental Specimens during Suspected Bioterror Attacks: Inhibitory Influences of Common Household Products▽

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After terrorists attacked the United States in 2001, the appearance of letters and other objects containing powdery substances with unknown potentials for biological threat focused attention on the speed, sensitivity, and reliability of diagnostic methods. This study summarizes the abilities and limitations of real-time PCR, electron microscopy (EM), and virus isolation when used to detect potential bioweapons. In particular, we investigated the inhibitory influences of different common household products present in environmental specimens on PCR yield, EM detection, and virus isolation. We used vaccinia virus as a model for orthopoxviruses by spiking it into specimens. In the second part of the study, we describe modifications of diagnostic methods to overcome inhibitory effects. A variety of PCR amplification enhancers, DNA extraction protocols, and applications of internal controls were evaluated to improve diagnostic simplicity, speed, and reliability. As a result, we strongly recommend using at least two different frontline techniques in parallel, e.g., EM and PCR. A positive result obtained by any one of these techniques should be followed by a biological method to confirm the putative diagnosis. Confirmatory methods include virus isolation followed by an agent-specific immunofluorescence assay to confirm the presence of replication-competent particles.

Following the anthrax postal attacks in the United States during the fall of 2001 (19), a surge of related hoax attacks was perpetrated against individuals and institutions around the world (7). In particular, letters and other objects containing powdery substances posed a potential biological threat and focused the attention of the public and of the clinical and scientific communities on the speed, sensitivity, and reliability of diagnostic techniques for detecting infectious agents in environmental specimens. Variola virus ("smallpox" virus; family *Poxviridae*, genus *Orthopoxvirus*), classified by the Centers for Disease Control and Prevention (CDC) as a category A biological agent (11), and genetically modified orthopoxviruses are potential bioweapons.

Letters and parcels containing powders are suspected sources for transmitting inhalable bioweapons (19). A variety of smallpox outbreak scenarios have been published, revealing a critical timeframe from the initial dispersal of the agent to the first patient diagnosis correlated to the attack (12, 24, 29, 36). The rapid and reliable detection and definitive identification of orthopoxviruses from an environmental specimen is crucial to help contain this situation and shorten the response time from several days when the first clinical signs develop to several hours, during which time potentially infected people could be isolated and treated.

Preanalysis procedures were generally well established after the bioterrorist attacks of 2001 (8). These included patient isolation and supportive care, appropriate sampling, specimen storage, and transport to a frontline diagnostic lab. Similar efforts were undertaken for all postanalysis steps, further improving the preparedness of responsible institutions for better containment and control of a suspected outbreak. A different situation was found for diagnostic methods applied to detect agents in environmental samples, where the nature of the specimen matrix was mostly unknown. Patient symptoms permit valuable insight into the etiologic agent present, whereas the content of an environmental specimen is largely a mystery, and the possibilities of different agents in different mixtures and combinations are infinite. It is therefore very difficult to properly evaluate diagnostic procedures for environmental samples.

Detection techniques must cover a broad spectrum of agents contained within a broad range of matrices while also being species specific and reliable. At present, molecular methods such as real-time PCR are the most extensively used techniques for the detection of infectious agents. PCR is rapid and sensitive and allows a high sample throughput and the further genetic characterization of the microorganism (26). However, when attempting to detect unknown infectious agents in environmental specimens, the selective species- or family-specific PCR approach alone is disadvantageous (31) and should therefore be complemented by additional frontline diagnostic techniques like electron microscopy (EM) (27, 30). The "open view" of EM allows an unbiased, rapid detection of viruses and other agents if sufficiently high particle concentrations are present (15, 16). Since EM and PCR cannot discriminate between infectious and noninfectious particles, a positive result obtained by one of these methods must be confirmed by additional methods like virus isolation in cell culture (23). Proof of replication-competent particles in environmental samples is

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required for an adequate risk assessment (German Smallpox Preparedness Plan, available from www.rki.de).

With the general understanding that environmental specimens contain inhibitors of reverse transcriptase and thermostable DNA polymerases, e.g., humic acid (45), several PCR assays for the diagnosis of suspected bioterror-related bacteria have been evaluated using complex matrices as found in soil or wastewater, for example (9, 10, 18, 38, 40). In contrast, most of the recently published PCR assays for the diagnosis of orthopoxviruses have been evaluated using only purified DNA, clinical samples, or standardized cell culture material (1, 13, 14, 21, 25, 32, 33, 35, 37, 39, 42–44). Therefore, to accurately examine environmental specimens, diagnostic methods have to be adapted to overcome potential inhibition. Besides the adjustment of the protocol, special attention has to be given to appropriate controls.

This study evaluates the abilities and limitations of real-time PCR, EM, and virus isolation for the detection of poxviruses in environmental samples. We compared the influences on and the consequences of the detection of infectious viral particles in the presence of inhibiting substances in environmental specimens. We used freeze-dried vaccinia virus (VACV) as a template model for variola virus mixed with 10 selected powdery household products to analyze their inhibitory effects. In the second part of the study, we concentrated on methods to overcome the inhibition while promoting easy handling, rapid turnaround time, and the production of reliable results. To achieve this, PCR enhancers, DNA extraction protocols, and the inclusion of internal amplification controls were evaluated.

MATERIALS AND METHODS

Virus stocks. VACV (strain NYCDH) was propagated in Hep2 cells (ATCC CCL-23) at a multiplicity of infection of 0.25 in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 5% fetal bovine serum and 1% glutamine at 37°C in a 5% CO_2 atmosphere. Infected cells were incubated for approximately 4 days until a pronounced cytopathic effect was observed. Supernatant and cells were harvested from infected cultures and subsequently separated by centrifugation (10 min at 1,000 × *g*) after freeze-thawing. The infectivity titers of VACV were determined using a plaque assay (17) on Vero E6 cells grown in DMEM supplemented with 10% fetal bovine serum. Virus titers were expressed as PFU per ml. Virus-containing supernatant was aliquoted and stored at −75°C until use.

Before each experiment, virus suspensions were diluted with DMEM to three different final concentrations: 1×10^9 PFU/ml (high virus concentration [HVC]), 1×10^6 PFU/ml (medium virus concentration [MVC]), and 1×10^3 PFU/ml (low virus concentration [LVC]). The MVC and LVC suspensions represented the hypothetical detection limits of EM and PCR per assay, respectively. Thereafter, aliquots of 0.5 ml of the different VACV suspensions were freeze-dried overnight at 4 Pa and −55°C.

Environmental substances. Ten different powdery common household products globally available were encoded with letters as substances A to J (powdered sugar, black pepper, washing detergent, flour, potting soil, baking powder, salt, tobacco, coffee powder, and black tea, respectively). These substances have been identified in suspected bioterror-related samples in recent years and partially proved to have inhibitory potentials. Depending on the solubility, 0.05 or 0.1 g of each substance was mixed with an aliquot of freeze-dried VACV to simulate the type of suspect packages received during the bioterrorist attacks of 2001. Each of the powdery mixtures was dissolved by vortexing in 0.5 ml of doubly distilled water. Thereafter, aliquots of 100 μl of the solutions were centrifuged at 3,000 × *g* for 5 min, and the amounts of model orthopoxviruses in supernatants were quantified using PCR, EM, and plaque assay. In general, each experiment was repeated once to qualitatively confirm the results.

EM. Negatively stained environmental specimens and controls were subjected to EM as described elsewhere (3). Briefly, 400-mesh copper grids covered with Pioloform F and carbon were floated on suspension drops, washed twice on drops of doubly distilled water, and then contrasted on a drop of 1% uranyl acetate (pH 4.0 to 4.5). Excess stain was drawn off with torn filter paper, and the grids were allowed to air dry. The specimens were examined in a transmission EM (Zeiss EM 10A) at a magnification of ×10,000 and an accelerating voltage of 80 kV for at least 15 min. Virus particles on 10 meshes were counted and final concentrations were determined as follows: a mean of 1 particle/10 meshes is equivalent to 1×10^6 particles/ml, 1 particle/mesh is equivalent to 1×10^7 particles/ml, and 10 particles/mesh is equivalent to 1×10^8 particles/ml. Only specimens with HVC and MVC were examined, since the LVC was below the detection limit of EM.

Real-time PCR. To evaluate inhibitory potentials in an orthopoxvirus-specific rpo18 assay (32), the susceptibility to error was investigated using known inhibitors as a control after mixing VACV DNA with 10-fold dilutions of lipopolysaccharide, lactoferrin, humic acid, dextran sulfate, and heparin zinc salt.

Additionally, VACV DNA from a spiked environmental specimen was isolated by a standard procedure (the QIAamp DNA blood mini kit [Qiagen, Hilden, Germany] method [QB]). All PCRs contained 10 pmol of each primer (TIB Molbiol, Berlin, Germany), 3 pmol of each hybridization probe (TIB Molbiol), 1 U of PlatinumTaq (Invitrogen, Karlsruhe, Germany), 5 mM Mg^{+2}, and 5 μl of template DNA. Primer sequences and cycling conditions were used as described previously (32), but to achieve a higher sample throughput, a TaqMan probe (orthopoxvirus rpo TM I, F-ATCgCTAAATgATACAgTACCC gAATCTCTACT P [F is for 6-carboxyfluorescein attached to the 5′ terminus, T is for 5-carboxytetramethylrhodamine attached to 5-ethylamino-D-thymidine, P is for phosphate, and lowercase letters indicate guanine]) assay was also applied. All real-time PCRs were performed using real-time PCR instruments of the 7700/7500/7000 sequence detection system series (Applied Biosystems, Foster City, CA).

Comparison of DNA extraction methods for environmental specimens. The following DNA extraction methods were compared for their abilities to remove inhibitors: QB, the Qiagen MagAttract DNA mini M48 kit (Qiagen, Hilden, Germany) method (QMA), the Invitrogen ChargeSwitch genomic DNA 0.2-to-1 ml blood kit (Invitrogen, Karlsruhe, Germany) method (InCS), phenol-chloroform extraction according to the method of Sambrook et al. (41), the SoilMaster DNA extraction kit (Epicenter Biotechnologies, Madison, WI) method (SoilM), and guanidinium thiocyanate-based extraction according to the work of Boom et al. (4–6).

Several PCR enhancers were tested for their abilities to compensate the inhibitory effect during template amplification: betaine (provided by GenExpress, Berlin, Germany), bovine serum albumin (Sigma-Aldrich, Germany), α-casein (Sigma-Aldrich, Germany), TaqMaster PCR enhancer (Eppendorf, Hamburg, Germany), and AmplyFly (GenExpress, Germany). Positive-control samples containing VACV only revealed cycle threshold (C_T) values between 19 and 21. Samples with C_T values over 40 in two separate measurements were rated negative, where the C_T is defined as the PCR cycle at which the gain in fluorescence generated by the accumulating amplicon exceeds 10 standard deviations of the mean baseline fluorescence, using data taken from cycles 3 to 15 (20).

Internal control. An internal control was established for environmental specimens by spiking with porcine parvovirus (PPV) and using a duplex PCR assay for rpo18 and PPV (RKI internal control [unpublished]). Other internal controls may be feasible; however, they need to be individually adapted to and evaluated for each assay. The possibility that competitive PCR reduced PCR performance and the occurrence of cross-reactivity were tested for both assays. Sensitivity was evaluated using different dilutions of VACV in DMEM with and without spiked PPV for both PCR assays individually and in duplex. Subsequent validation was accomplished by Probit analysis.

RESULTS

Plaque assay. After freeze-drying, the control specimen containing VACV produced virus titers of 2×10^9 PFU/ml (HVC), 10^6 PFU/ml (MVC), and 4×10^2 PFU/ml (LVC). Plaques of VACV mixed with washing detergent were undetectable at any virus concentration due to the toxic effect of the substance (Table 1). The presence of black tea resulted in a 10^5-fold reduction of VACV from what was seen for the HVC control, while both the MVC and LVC controls were negative, producing no visible cytotoxic effects. No viral replication could be detected for three other specimens (black pepper, tobacco, and coffee powder).

TABLE 1. Comparison of plaque assay, EM, and PCR for testing VACV mixed with 10 different household products[a]

Sample	Result of indicated test at:							
	HVC			MVC			LVC	
	PA[c]	EM	PCR	PA[c]	EM	PCR	PA[c]	PCR
Co[b]	++	++	++	++	++	++	++	++
Powdered sugar	++	++	++	++	++	++	++	−−
Black pepper	++	++	++	++	−−	++	−−	++
Washing detergent	−−	−+	++	−−	−−	−−	−−	−−
Flour	++	++	++	++	−+	++	++	++
Potting soil	++	++	−−	++	++	−−	++	−−
Baking powder	++	++	++	++	++	−−	++	−−
Salt	++	++	++	++	++	++	++	−−
Tobacco	++	++	++	++	−−	++	−−	−−
Coffee powder	++	++	++	++	−+	++	−−	++
Black tea	++	++	++	−−	++	−−	−−	−−

[a] VACV was used in HVC, MVC, and LVC. ++, positive diagnostic results; −−, negative result; −+, equivocal diagnosis because of atypical VACV structure when viewed by EM. EM was not performed for LCV, since this concentration is below its detection limit.
[b] Co, VACV without additional substance.
[c] PA, plaque assay (infectious VACV).

EM. Control specimens containing VACV without additives produced virus titers of approximately 2×10^8 virus particles/ml (HVC) and 2×10^6 viruses/ml (MVC). The presence of five substances (black pepper, washing detergent, flour, tobacco, and coffee powder) resulted in negative interference with our EM preparation (Table 1).

In combination with washing detergent, virus lysis and reduced virus adsorption to the EM grid resulted in a sensitivity approximately 100- to 1,000-fold lower than that for the control. Additionally, the only particles visible had visibly disrupted membranes (Fig. 1b). It was not possible to detect particles in the MVC control. In combination with flour, virus adsorption to the EM grid was 10 times lower than with the HVC control. Coffee powder also adversely influenced virus morphology (Fig. 1c). The typical surface structures of the particles were obscured, so that particles could be identified only partially, based on their diameter. Furthermore, it was not possible to detect particles in two additional substances (black pepper and tobacco) at MVC.

Real-time PCR. To evaluate the sensitivity of the orthopoxvirus-specific PCR assay to inhibition, different PCR inhibitors were applied in 10-fold dilutions. Complete PCR inhibition was achieved with lipopolysaccharide at 100 μg/ml, lactoferrin and humic acid at 1 μg/ml, dextran sulfate at 100 ng/ml, and heparin zinc salt at 5 U/ml.

Control specimens containing VACV in DMEM revealed virus concentrations of 4×10^8 genome equivalents (GE)/sample (HVC), 6×10^5 GE/sample (MVC), and 1×10^4 GE/sample (LVC). In tests of spiked environmental specimens, potting soil completely inhibited PCR amplification from spiked environmental specimens at all virus concentrations (Table 1). An additional three substances (washing detergent, baking powder, and black tea) inhibited amplification at MVC and LVC, and VACV DNA could not be amplified in specimens containing powdered sugar, salt, or tobacco at LVC.

Comparison of DNA extraction methods for environmental specimens. We next applied different DNA extraction methods to eliminate inhibitors of the standard PCR protocol. Table 2 shows the influence of the extraction on the PCR. The PCR detection of VACV in undiluted DNA extracts failed in the presence of at least one substance regardless of the extraction method. The most effective extraction methods proved to be

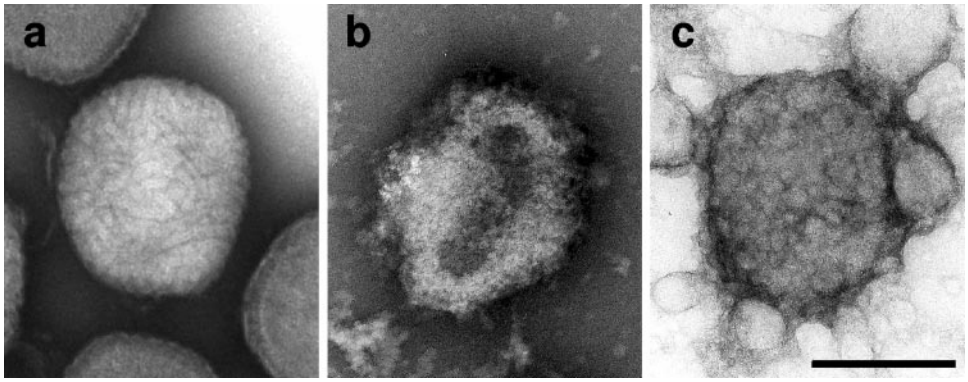

FIG. 1. EM diagnosis of VACV particles. Comparison of virus morphologies after incubation of VACV with different household products. (a) Typical appearance of VACV: brick-shaped with short surface projections. Virion with unstable membrane in combination with washing detergent (b) or with covered surface structures in combination with coffee powder (c); both combinations make it very difficult to differentiate between possible virus particles and background structures. Negative staining with 1% uranyl acetate. Bar = 100 nm.

TABLE 2. Comparison of different DNA extraction methods[a]

Sample	Result by indicated method					
	QB	QMA	InCS	Phenol	Boom	SoilM
Co[b]	+/+	+/+	+/+	+/+	+/+	+/+
Powdered sugar	+/+	+/+	+/+	+/+	+/+	+/+
Black pepper	+/+	+/+	−/+	−/+	+/+	+/+
Washing detergent	−/+	−/−	−/−	−/+	+/+	+/+
Flour	+/+	+/+	+/+	+/+	+/+	+/+
Potting soil	−/+	+/+	−/−	−/+	−/+	−/−
Baking powder	−/−	+/+	+/+	−/−	+/+	+/+
Salt	+/+	+/+	+/+	+/+	+/+	+/+
Tobacco	+/+	+/+	−/+	−/+	+/+	+/+
Coffee powder	+/+	+/+	−/+	−/+	+/+	+/+
Black tea	−/+	−/−	−/−	−/+	+/+	+/+

[a] After the indicated DNA extraction method was applied, PCR was used to amplify from extracts of mixtures containing VACV and various inhibiting household substances. Target VACV DNA was prepared according to the manufacturer's instructions or by use of standard protocols and DNA samples were subjected to real-time PCR undiluted or in 1:10 dilutions. All samples analyzed by PCR with C_T values of up to 40 in two separate measurements were rated positive. +/+, positive PCR results for undiluted and diluted samples; −/−, negative PCR results for undiluted and diluted samples; −/+, negative result for undiluted sample but positive result for diluted sample.

[b] Co, VACV control without additional substance.

TABLE 3. Comparison of SoilM and the Boom method[a]

Sample and method	PCR result for extract			
	Undiluted plus:		Diluted 1:10 plus:	
	1 U Taq	5 U Taq	1 U Taq	5 U Taq
Co[b]	+	+	+	+
Sample E (potting soil) as assessed by:				
Boom method	−	+	+	+
SoilM	−	−	−	+

[a] VACV DNA was extracted from sample E (potting soil) and subjected to real-time PCR. Extracts were either undiluted or diluted 1:10 and reaction mixtures contained either 1 U *Taq* DNA polymerase or 5 U *Taq* DNA polymerase. All samples analyzed by PCR with C_T values of up to 40 in two separate measurements were rated positive. +, Positive PCR result; −, negative PCR result.

[b] Co, VACV control without additional substance; DNA was extracted using both SoilM and the Boom method.

QMA, SoilM, and the Boom method, with positive results for 9 out of 10 extracts. The success rate could be improved by diluting the specimen 1:10 after applying the respective extraction method (Table 2). Only potting soil, baking powder, and black tea proved to be difficult for InCS/SoilM, QB/phenol, and QMA/InCS, respectively. Suspected PCR-positive results with C_T values of about 38 were obtained for washing detergent when applying QMA and InCS, which would routinely imply a follow-up confirmatory PCR run. Interestingly, a PCR sensitivity similar to that for the control could be achieved only by use of a 1:10 dilution of DNA extracted by the Boom method. The PCR protocol could be further improved by the addition of 4 units of DNA polymerase, resulting in the successful amplification of all undiluted Boom method-extracted specimens (Table 3). Commercially available PCR enhancers (betaine, bovine serum albumin, casein, Eppendorf HotMaster, Invitrogen AmplyFly) showed no reproducible PCR improvement when employed to counter the effects of the different inhibitors (data not shown).

An internal control for the orthopoxvirus-specific rpo18 assay was established to identify false-negative results. The addition of about 40 GE of PPV prior to purification enabled the control of both DNA extraction and template amplification by PCR. PPV is not pathogenic for humans. Furthermore, the PPV assay is not used for the routine diagnosis of suspected bioterror-related specimens and therefore presents no additional risk for laboratory contamination. Under the PCR conditions established for the rpo18 assay, no competitive influence between the two assays was observed. Furthermore, no loss of sensitivity was observed for the duplex PCR compared with both monoplex assays. To evaluate the detection limit of the duplex PCR, a Probit analysis was performed by repetition of the detection of VACV ($n = 8$) in the presence of ~50 PPV infectious particles. Real-time PCR analysis showed that the duplex assay was able to detect 15 PFU of VACV with a confidence interval of 95%.

DISCUSSION

The release of mixtures of single or multiple virus species together with powdery substances and their use for bioterrorism is a topic of active discussions (19). Before a suspect package or specimen is subjected to diagnostic procedures, a risk assessment is completed to determine the impact of the powdery substance's particle size and capacity to desiccate. Environmental specimens behave differently from clinical specimens during specimen preparation and are often examined with specifically evaluated methods. However, there are ways to circumvent the inhibitory effects associated with these specimens by specifically adjusting the diagnostic procedure and controlling the sensitivity and specificity of the PCR protocol.

We examined the environmental specimen preparation techniques we use for screening material suspected of containing bioweapons. Ten substances chosen from letter contents received in previous years were examined to evaluate our standard procedures (PCR, EM, and virus isolation). We determined the possibility of failure for all three approaches by adding different commonly available household products. Despite the regular use of PCR, EM, and virus isolation protocols for examining clinical specimens and the extensive evaluation of these methods, we found it impossible to employ these protocols for the examination of environmental specimens without some loss of sensitivity. As demonstrated in Table 1, several substances were able to completely inhibit at least one diagnostic approach. The plaque assay was vulnerable to both cytotoxic substances (washing detergent) and substances reducing virus infectivity in solution (black tea). Despite VACV being rendered noninfectious in solution with washing detergent, one cannot exclude the possibility that VACV remains infectious within a dried mixture of the substance. To circumvent the influence of inhibiting substances, an application of combined plaque assay and subsequent real-time PCR could be employed, as previously demonstrated by Nitsche et al., who found an extremely low detection limit of 3 PFU of orthopoxvirus in less than 5 hours (34). This allowed the dilution of the environmental specimen by several orders of magnitude after low-speed centrifugation, resulting in the dilution of the inhib-

itor(s) while maintaining the viral load at detectable levels. Furthermore, no special safety precautions needed to be considered, since the incubation was stopped before the assembly of infectious viruses was complete.

EM results largely depend on the original virus concentration within the specimen, which must be higher than 10^5 to 10^6 particles/ml (3). Furthermore, despite the use of adherent grids after pretreatment with Alcian blue or glow discharge, some substances can reduce the adsorption of viruses to the grid. Other substances can detrimentally influence virus morphology. When examining samples suspected of containing virus, we propose a preanalytical estimate of possible inhibition by spiking the specimen into a parallel preparation of the suspect substance with a defined amount of a known and easily diagnosed virus, e.g., 10^9 particles/ml of adenovirus. Such an internal control will guarantee an adequate assessment of the virus adsorption to the grid as well as the influence on the structural conservation of virus particles.

Summarizing the PCR results, the orthopoxvirus-specific rpo18 assay could be completely abrogated by inhibitors at concentrations previously described for other PCR assays (2, 28, 45). As demonstrated, the validity of PCR results was improved by using a PPV internal control. Mixing some of the tested household products with VACV also resulted in complete inhibition for a variety of DNA extraction methods, but we observed that different extraction methods were more or less efficient at preparing amplifiable DNA from different substances.

In contrast to the wide use of QB as a standard extraction method for clinical specimens, the extraction according to the Boom method proved to be less prone to carry-through inhibition and permitted more-reliable results. Furthermore, inhibitory effects could be diminished in concentrated samples by diluting the examined specimen DNA 10-fold, which has already been demonstrated for monkeypox virus by Kulesh et al. (22). Similar results could be obtained by increasing the amount of polymerase fivefold. SoilMaster is specifically described for DNA extraction from environmental samples but failed to eliminate inhibitors in one sample. Successful amplification resulted only after the addition of polymerase in combination with the dilution of the VACV DNA. Otherwise, SoilMaster proved to be one of the most effective extraction kits but is unsuitable for mobile applications, since both a tabletop microcentrifuge and an ice bath are necessary for DNA extraction. Additionally, the 55-min time period required is relatively lengthy compared to approximately 25 min for the Boom method. It is noteworthy that the addition of so-called PCR enhancers could not consistently reduce inhibition.

Taken together, our results lead us to strongly recommend using at least two frontline techniques in parallel, e.g., EM and PCR. For EM, we recommend applying properly inactivated specimen to Alcian blue-treated grids, negatively contrasted with two different stains, e.g., 1% uranyl acetate and 2% phosphotungstic acid. A parallel sample spiked with a standard high-titer preparation of a different, well-characterized agent should be used to investigate the adsorption step and the specimen quality.

For PCR, DNA from suspect specimens should be extracted according to the Boom method, and subsequently both undiluted and 1:10 diluted extract should be amplified with 5 units of polymerase with and without an internal virus control. Further research in related areas may strengthen our recommendations.

Applying EM and real-time PCR as frontline techniques after the specimen arrives at the laboratory will permit diagnoses after approximately 30 min and 4 h, respectively. The PCR process can be further shortened to about 2 h by use using a high-speed real-time PCR cycler. In the case of a positive result, virus isolation of the suspected agent should be attempted. A positive virus isolation can be confirmed in the shortest time possible by detecting viral RNA after reverse transcriptase PCR amplification. This will add another 2 hours for the cultivation plus an additional 2 hours for the repeated PCR (34). In total, including specimen assessment, fixation, and observation, a final diagnosis and risk assessment can be accomplished within approximately 8 h, permitting a rapid response and therefore improving the likelihood of controlling a bioterror attack.

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